



Effects of Clavulanic Acid Treatment on Reinstatement to Methamphetamine, Glial Glutamate Transporters, and mGluR 2/3 Expression in P Rats Exposed to Ethanol

Yusuf S. Althobaiti^{1,2} · Fahad S. Alshehri¹ · Alqassem Y. Hakami¹ · Alaa M. Hammad¹ · Youssef Sari¹ 

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Abstract

Evidence demonstrated that the glutamatergic system is implicated in mediating relapse to several drugs of abuse, including methamphetamine (METH). Glutamate homeostasis is maintained by a number of glutamate transporters, such as glutamate transporter type 1 (GLT-1), cystine/glutamate transporter (xCT), and glutamate aspartate transporter (GLAST). In addition, group II metabotropic glutamate receptors (mGluR2/3) were found to be implicated in relapse-seeking behavior. Ample evidence showed that β -lactam antibiotics are effective in upregulating GLT-1 and xCT expression, thus improving glutamate homeostasis and attenuating relapse to drugs of abuse. In this study, we investigated the reinstatement of METH using conditioned place preference (CPP) in male alcohol-preferring (P) rats exposed to home-cage free choice ethanol drinking. Here, we tested the effect of clavulanic acid (CA), a β -lactam, on the reinstatement of METH-seeking and ethanol drinking. In addition, we examined the expression of GLT-1, xCT, and GLAST as well as metabotropic glutamate receptor (mGluR2/3) in the nucleus accumbens (NAc) shell, NAc core, and dorsomedial prefrontal cortex (dmPFC). A priming i.p. injection of METH reinstated preference in METH-paired chamber following extinction. Chronic exposure to ethanol decreased the expression of GLT-1 and xCT in the NAc shell, but not in the NAc core or dmPFC. CA treatment blocked the reinstatement of METH-seeking, decreased ethanol intake, and restored the expression of GLT-1 and xCT in the NAc shell. In addition, the expression of mGluR2/3 was increased by CA treatment in the NAc shell and dmPFC. These findings suggest that these glutamate transporters and mGluR2/3 might be potential therapeutic targets for the attenuation of reinstatement to METH-seeking.

Keywords Methamphetamine · Glutamatergic transporters · Conditioned place preference · mGluR2/3 · Clavulanic acid

Introduction

Ample evidence suggests that ethanol use disorder is exceptionally high in amphetamine-dependent subjects (Stinson et al. 2005). Methamphetamine (METH) users frequently use ethanol (Bujarski et al. 2014; Furr et al. 2000), which may result in several deteriorating effects (for a review, see ref. (Althobaiti and Sari 2016)). Despite the evidence of the high rate of the co-abuse of METH and ethanol, little is known

about the effects of co-exposure of these drugs or METH on ethanol drinking in rodent models. However, a recent study showed that METH self-administration decreased ethanol intake (Winkler et al. 2016). In this study, we examined the effects of METH co-exposure for seven consecutive days on home-cage voluntary ethanol intake in alcohol-preferring (P) rats and the role of the glutamatergic system in this drug co-exposure animal model.

One of the main brain regions involved in the rewarding and reinforcing effects of drugs of abuse is the nucleus accumbens (NAc) (Bardo 1998; Wise and Rompré 1989). Glutamatergic neurotransmission in the NAc plays a critical role in the relapse behavior to different drugs of abuse. Importantly, deficits in glutamate clearance in the NAc have been found to be associated with chronic drug use and drug-seeking behavior (Das et al. 2015; Fujio et al. 2005; Knackstedt et al. 2010; Melendez et al. 2005). The NAc receives glutamatergic inputs from the prefrontal cortex (PFC) as well as other brain regions (Kelley et al. 1981; Phillipson and Griffiths 1985). Dorsomedial PFC

✉ Youssef Sari
youssef.sari@utoledo.edu

¹ College of Pharmacy and Pharmaceutical Sciences, Department of Pharmacology and Experimental Therapeutics, University of Toledo, Health Science Campus, 3000 Arlington Avenue, HEB 282G, Toledo, OH 43614, USA

² College of Pharmacy, Department of Pharmacology and Toxicology, Taif University, Taif, Saudi Arabia

(dmPFC), in particular, has been implicated in mediating the reinstatement of cocaine-seeking (Berglind et al. 2009; McLaughlin and See 2003). Glutamate release from dorsal PFC projections to the NAc core has been found to mediate the reinstatement of cocaine-seeking (McFarland et al. 2003; McFarland et al. 2004). Of note, studies demonstrated that blockade of metabotropic glutamate receptor 1 (mGluR1) and mGluR5 as well as activation of mGluR2/3 in central reward brain regions can decrease the reinstatement to several drugs of abuse (Bossert et al. 2006; Cornish and Kalivas 2000; Dravolina et al. 2007; Lee et al. 2005; McFarland et al. 2004; Tessari et al. 2004). Therefore, modulating the glutamatergic system at the synaptic and extra-synaptic receptors might be an effective strategy for attenuating reinstatement of drug-seeking.

Glutamate homeostasis is maintained by several glutamate transporters, including glutamate transporter type 1 (GLT-1, its human homolog excitatory amino acid 2, EAAT2), cystine/glutamate transporter (xCT), and glutamate aspartate transporter (GLAST). GLT-1 clears about 90% of extracellular glutamate and is frequently reported to be downregulated in the NAc following exposure to drugs of abuse (Fischer et al. 2013; Knackstedt et al. 2009; Rothstein et al. 1995; Sari and Sreemantula 2012; Shen et al. 2014). Furthermore, xCT was also downregulated in the NAc following cocaine withdrawal (Baker et al. 2003). In fact, decrease in the xCT expression in the NAc, which has a significant role in regulating glutamate homeostasis (Alhaddad et al. 2014; Baker et al. 2002), is associated with decrease in basal glutamate concentrations and subsequent impairment of the activation of mGluR2/3. Decrease in glutamatergic tone on mGluR2/3 can lead to an increase in glutamate release in the NAc during reinstatement (Dietrich et al. 2002; LaLumiere and Kalivas 2008; Madayag et al. 2007; Moran et al. 2005).

Importantly, treatment with ceftriaxone (CEF), a known GLT-1 upregulator, has been reported to prevent the reinstatement of METH-, cocaine-, ethanol-, nicotine-, and heroin-seeking (Abulseoud et al. 2012; Alajaji et al. 2013; Knackstedt et al. 2010; Qrunfleh et al. 2013; Sari et al. 2009; Shen et al. 2014). Moreover, our lab recently showed that METH exposure could decrease the expression of GLT-1 in the NAc. In addition, chronic ethanol drinking was shown to decrease GLT-1 and xCT expression in the NAc (Alhaddad et al. 2014; Das et al. 2015). Therefore, CEF treatment decreased ethanol intake and upregulated GLT-1 in the NAc and PFC (Alhaddad et al. 2014; Althobaiti et al. 2016; Sari et al. 2011, 2013). CEF's upregulatory effect on GLT-1 expression is suggested to be mainly attributed to the β -lactam ring found in this drug and other β -lactam antibiotics (Rothstein et al. 2005). Thus, clavulanic acid (CA), which is a non-antibiotic β -lactam compound with desirable therapeutic profile relative to CEF, could be a better alternative. In fact, CA is orally available, relatively safe, and can cross the blood-brain barrier (Münch et al. 1981; Nakagawa et al. 1994). Importantly, CA (1 mg/kg

and 10 mg/kg, i.p) was shown to decrease cocaine self-administration in part through upregulation of GLT-1 in the NAc (Kim et al. 2016). Also, CA (5 mg/kg) was reported to decrease chronic ethanol drinking, which was associated with an increase in GLT-1 and xCT expression in the NAc and PFC in P rats (Hakami and Sari 2017).

Therefore, we examined the CA effect on the reinstatement to METH in a conditioned place preference (CPP) paradigm in P rats that had free access to ethanol. P rats were used in this study as an established animal model of alcoholism since these rats can drink 5–8 g ethanol/kg/day voluntarily (Li et al. 1993). Moreover, according to previous reports, the reinforcing effects of stimulants, such as cocaine, were more pronounced in P rats as compared to Wistar rats (Katner et al. 2011). P rats have also been reported to be less sensitive to the sedating effects of ethanol (for a review, see ref. (McBride and Li 1998)). This tolerance to the sedating effects of ethanol makes P rats a good animal model for testing ethanol and METH co-abuse using the CPP procedure. Of note, the CPP has been an important alternative procedure to self-administration in investigating reward and relapse of several drugs of abuse (for a review, see ref. (Tzschentke 1998)). In this paradigm, the drug of abuse is associated with specific environmental cues. As a result, when the drug is rewarding, animals prefer to be in contact with the drug-paired environment (for a review, see ref. (Carr et al. 1989)). Importantly, CPP has been used for all drugs of abuse, including ethanol, opiates (such as heroin, morphine, and methadone), and stimulants (such as cocaine and METH) (Ashby et al. 2003; Mueller and Stewart 2000; Reid et al. 1985; Steinpreis et al. 1996; Tzschentke and Schmidt 1995; Zakharova et al. 2009).

Also, we investigated the effect of METH on ethanol drinking and the effect of co-exposure of these drugs on locomotor activity in the P rat model. We also tested the effects of CA on ethanol intake in P rats pre-exposed to METH for seven consecutive days. Finally, to explore the possible role of the glutamatergic system, we investigated the expression of different glutamate transporters (GLT-1, xCT, and GLAST) and mGluR2/3 in the NAc shell and core, as well as the dmPFC.

Materials and Methods

Animals

Male P rats, at the age of 21–30 days, were obtained from the Indiana University Medical Center (Indianapolis, IN) Indiana Alcohol Research Center breeding colonies. Animals were single-housed in standard plastic cages with a controlled temperature (21 °C) and humidity (30%) on 12:12 light-dark cycle and had free access to food, and a three-bottle drinking paradigm (water and two ethanol concentrations (15 and 30% v/v)). Rats had ad libitum food, water, and ethanol throughout the

experimental procedure, except for the control (water group) which did not have access to ethanol. Animal experimental procedures were approved by the Institutional Animal Care and Use Committee of The University of Toledo in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institutes of Health and the Guide for the Care and Use of Laboratory Animals.

Drugs

(+)-METH hydrochloride and CA were purchased from Sigma-Aldrich (St. Louis, MO). Saline solution (0.9% NaCl) was used to dissolve both drugs. Ethanol (95%; Decon Labs, Inc.) was diluted in water.

Ethanol Drinking Paradigm

Rats had free access to a three-bottle drinking paradigm (two ethanol concentrations (15 and 30% v/v) and water) and food, except for the control (water group) which did not have access to ethanol, for 5 weeks before CPP testing. Ethanol intake, water intake, and body weight were measured three times a week during weeks 4 and 5, and the average values of these two weeks were considered as the baseline. The measurements of fluid intake and body weight were then recorded daily throughout the experiment.

Experimental Design

Experimental groups and schedule are illustrated in Table 1. A separate group of rats was utilized to investigate whether CA has any effect on CPP. These rats were treated with CA (5 mg/kg, i.p.) and underwent the same procedure as described below in the conditioning phase. After completion of the seven conditioning days, rats were tested for preference. During week 6, the CPP experiment was initiated, in which each group received either saline or METH (2.5 mg/kg, i.p.) injections for 7 days (conditioning phase), followed by 7 days treatment with either saline or CA (5 mg/kg, i.p.) (extinction phase). Finally, rats were tested for reinstatement produced by an i.p. injection of METH (2.5 mg/kg). A one water control group and three ethanol-exposed groups have been used in this study as follows: (a) the control (water group) group had free access to water and

food throughout the whole experiment and received saline i.p. injections in conditioning, extinction, and reinstatement phases; (b) the Saline-Saline group had free access to ethanol (15 and 30% v/v), as well as ad libitum food and water throughout the experiment. Rats received saline i.p. injections in conditioning, extinction and reinstatement phases; (c) the METH-Saline group had free access to ethanol (15 and 30% v/v), as well as ad libitum food and water throughout the experiment. Rats received METH i.p. injections in conditioning, saline in extinction, and METH in the reinstatement phase; and (d) the METH-CA group had free access to ethanol (15 and 30% v/v), as well as ad libitum food and water throughout the experiment. Rats received METH in conditioning, CA in extinction, and METH in the reinstatement phase.

Conditioned Place Preference Paradigm

Apparatus

A three-chamber CPP apparatus made of Plexiglas was used in this study. Two equal-sized chambers were distinguished by both visual and tactile cues (40 cm × 40 cm × 40 cm), and one smaller middle chamber (30 cm × 40 cm × 40 cm). The inner walls of the first chamber are white with horizontal black stripes and textured black floor (chamber 1). The inner walls of chamber 2 are black with vertical white stripes and smooth white floor. The middle chamber is featureless and separated from the two chambers by two guillotine doors. The CPP experiment was conducted according to previous studies in four distinct phases (Abulseoud et al. 2012; Arezoomandan et al. 2016).

The Habituation Phase

The first day of habituation was considered as day 1. On days 1–3, each rat was placed in the middle chamber with both doors closed for 3 min. Both doors were then opened, and the rats had free access to explore the entire apparatus for 20 min. On day 3, the test was recorded by a digital camera fixed above the apparatus (CPP Test). The time spent in each chamber was measured by an observer who was blinded to the experimental design. The locomotor activity was analyzed using the ANY-maze video tracking system. Rats were excluded from the study when they showed strong initial preference to any chamber for more than

Table 1 Experimental groups and schedule

	Group	Post-conditioning	Extinction	Reinstatement
	Control (water group)	Saline	Saline	Saline
Ethanol-exposed groups	CA group	CA or Saline	–	–
	Saline-Saline	Saline	Saline	Saline
	METH-Saline	METH or Saline	Saline	METH
	METH-CA	METH or Saline	CA or Saline	METH

67% of the total time as conducted previously (Alshehri et al. 2018; Fujio et al. 2005; Hammad et al. 2017).

The Conditioning Phase

The conditioning phase (days 4–10) consisted of 14 conditioning sessions conducted on seven consecutive days (morning and afternoon sessions). Each rat received i.p. injections of either 2.5 mg/kg METH or saline, and was placed in the corresponding chamber with the door closed for 20 min in the morning session. In the afternoon session (~ 5 h after the morning session), each rat received saline or 2.5 mg/kg METH i.p. and was placed in the opposite chamber with the door closed for 20 min. Since initial time spent in chamber 1, chamber 2, and the middle chamber was not significantly different, an unbiased design was utilized. Thus, random assignment of rats was utilized in which half of the animals received METH in chamber 1, and the other half received the drug in chamber 2. The control group received saline in both morning and afternoon sessions, and placed in either chambers (i.e., in chamber 1 in the morning and chamber 2 in the afternoon session) with doors closed for 20 min. The order of METH and saline administration was counterbalanced. METH was administered in the morning for half of the group of rats and saline for the other group of rats in the afternoon. On the following day, saline was administered to the same group of rats in the morning and METH was given in the afternoon, and the pattern was reversed for the other half of group of rats. Similarly, the orientation of the chambers was alternated as described in a previous study (Cunningham et al. 2006). On day 11, the CPP test was performed again as described previously. Animals were conditioned twice a day based on a previous report (Abulseoud et al. 2012).

The Extinction Phase

This phase is similar to the conditioning phase except for the rats that received METH in the conditioning phase were randomly assigned to receive either CA (5 mg/kg, i.p.) or saline (1 ml/kg) instead of 2.5 mg/kg METH (days 12–18). The dose of CA (5 mg/kg, i.p.) was chosen based on previous studies (Kim et al. 2016; Schroeder et al. 2014). The control group received saline in morning and afternoon sessions as described in the conditioning phase. On day 19, another CPP test was performed. Rats must show 25% reduction in time spent in the METH-paired chamber following the extinction training to be considered extinguished as described in previous work (Abulseoud et al. 2012).

Reinstatement Phase

On the morning of day 20, each rat was administered a single dose of either METH (2.5 mg/kg) or saline and placed in the corresponding chamber with the door closed for 20 min. In the

afternoon, saline or METH was administered (i.p.) and the rat was placed in the opposite chamber. On day 21, each rat was tested for CPP as described above.

Brain Tissue Harvesting

Rats were rapidly euthanized following reinstatement using the CPP test by CO₂ inhalation and immediately decapitated. Brains were then removed and rapidly frozen on dry ice and stored at – 80 °C. Brain sectioning and micro-punch procedure were performed as described previously (Sari and Sreemantula 2012). The NAc shell and core, and dmPFC were identified using stereotaxic according to The Rat Brain Atlas as shown in Fig. 1 (Paxinos and Watson 2007).

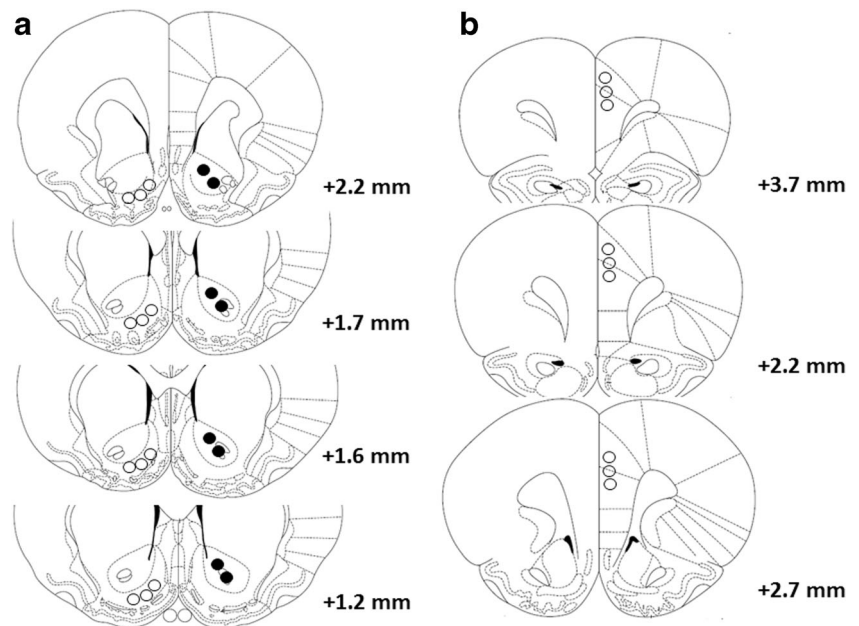
Western Blot

Western blot procedure was performed as previously described (Sari et al. 2009). Briefly, brain tissue was lysed in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1% Triton, 0.1% SDS) containing protease inhibitor cocktail. Extracted proteins were loaded onto 10–20% Tris-glycine gel. After separation, proteins were transferred from the gel onto a polyvinylidene difluoride (PVDF) membrane. The membrane was then blocked using 3% milk in Tris-buffered saline Tween 20 for 30 min. Guinea pig anti-GLT1 (Millipore Bioscience Research Reagents; 1:5000 dilution), rabbit anti-xCT antibody (Abcam; 1:1000 dilution), rabbit anti-mGluR2/3 antibody (Upstate Biotechnology; 1:1000 dilution), rabbit anti-GLAST (Abcam; 1:5000 dilution), or mouse anti β -tubulin antibody (Covance; 1:5000 dilution) was then added to the blocking buffer, and the membrane was incubated overnight at 4 °C. The membrane was then washed and incubated with horseradish peroxidase-labeled (HRP) anti-Guinea pig, anti-rabbit, or anti-mouse secondary antibody (1:5000). Chemiluminescent kit (SuperSignal West Pico) was used to incubate the membrane for protein detection. The membrane was exposed to Kodak BioMax MR films (Thermo Fisher Scientific). Films were then developed using an SRX-101A machine. Blots for each detected protein were digitized and quantified using an MCID system. Data were calculated as ratios of GLT-1/ β -tubulin, xCT/ β -tubulin, GLAST/ β -tubulin, and mGluR2/3/ β -tubulin relative to the control group (100% control-value). In each gel run, control (water group) was set as 100% and the expression of protein of interest for the other ethanol-exposed groups was computed relative to it as described in previous studies (Hammad et al. 2017; Li et al. 2003; Miller et al. 2008; Raval et al. 2003; Zhang and Tan 2011).

Statistical Analysis

Time spent in conditioning chambers (preference) and locomotor activity were analyzed using two-way repeated

Fig. 1 Representative micro-punch of brain regions: NAc shell (open circles) and core (closed circles) (a), and dmPFC (b). Numbers indicate the anteroposterior distance from the bregma according to The Rat Brain Atlas (Paxinos and Watson 2007)



measures ANOVA, followed by Newman-Keuls as post hoc test. In case of locomotion, distance traveled in the whole apparatus was measured at two different time-points (baseline and post-conditioning) in response to different treatments (control, ethanol-saline, and ethanol-METH). In case of preference, chambers represent the place that specific treatment was given (saline or METH) and time spent in each chamber was tested at different time-points (preconditioning, post-conditioning, extinction, and reinstatement), and the statistical analysis was conducted in the METH-Saline group as well as the METH-CA group.

Average ethanol intake, ethanol preference, water intake, and body weight were analyzed using two-way repeated measures ANOVA, followed by Bonferroni's multiple comparisons post hoc test. Immunoblot data were analyzed using one-way ANOVA, followed by Newman-Keuls as a post hoc test. GraphPad Prism was used to analyze all data. The p values were set at < 0.05 level of significance.

Results

Ethanol Drinking and Preference As Well as Water Intake and Body Weight

Effect of METH during the Conditioning Phase

We tested the effect of METH during the conditioning phase (conditioning 1–7) on ethanol intake and preference, water intake, and body weight. Statistical analysis using two-way repeated measures ANOVA revealed a significant effect of time ($F(7, 182) = 11.43, p < 0.0001$), treatment ($F(1, 26) = 11.01,$

$p = 0.0027$), and a significant interaction between time and treatment ($F(7, 182) = 5.372, p < 0.0001$). Bonferroni's multiple comparisons revealed that METH significantly decreased the average ethanol intake during days 1, 3, 4, and 5 of conditioning as compared to the saline treatment group ($p < 0.05, 0.0001, 0.05, \text{ and } 0.05$, respectively; Fig. 2a). No significant change in ethanol intake between METH and saline treatment groups was found during days 2, 6, and 7 of the conditioning phase.

We further investigated the effect of METH on ethanol preference. Two-way repeated measures ANOVA revealed a significant effect of time ($F(7, 182) = 7.597, p < 0.0001$), treatment ($F(1, 26) = 11.29, p = 0.0024$), and a significant interaction between time and treatment ($F(7, 182) = 3.956, p < 0.0005$). Bonferroni's multiple comparisons revealed that METH significantly decreased ethanol preference during day 3 ($p < 0.01$), day 4 ($p < 0.05$), day 5 ($p < 0.01$), and day 6 ($p < 0.05$) of conditioning as compared to saline treatment (Fig. 2b). No significant change was found in ethanol preference during day, 1, 2, and 7 of the conditioning phase between METH and saline treatment groups.

Two-way repeated measures ANOVA were conducted on water intake, and revealed a significant effect of time ($F(7, 182) = 2.171, p = 0.0387$), treatment ($F(1, 26) = 13.34, p = 0.0011$), and no significant interaction between time and treatment ($F(7, 182) = 1.416, p = 0.2014$). Bonferroni's multiple comparisons revealed that METH significantly increased the average water intake in day 1 ($p < 0.05$), day 2 ($p < 0.01$), and day 4 ($p < 0.05$) of conditioning as compared to saline group (Fig. 2c). No significant change was found in ethanol intake during days 3, 5, 6, and 7 of the conditioning phase between METH and saline treatment groups. A significant effect of

time ($F(7, 182) = 24.27, p < 0.0001$), no significant effect of treatment ($F(1, 26) = 3.676, p = 0.0662$), and a significant interaction between time and treatment ($F(7, 182) = 10.89, p < 0.0001$) were revealed on body weight using two-way repeated measures ANOVA. Bonferroni's multiple comparisons test did not reveal any significant change in body weight following METH treatment in all conditioning days as compared to saline group ($p > 0.05$; Fig. 2d).

Effect of CA during the Extinction Phase

We tested the effect of CA during the extinction phase (extinction 1–7) on ethanol intake and preference as well as water intake and body weight. Statistical analysis using two-way repeated measures ANOVA revealed a no significant effect of time ($F(7, 175) = 1.027, p = 0.4141$), a significant effect of treatment ($F(2, 25) = 18.14, p < 0.0001$), and a significant interaction between time and treatment ($F(14, 175) = 4.333, p < 0.0001$). Bonferroni's multiple comparisons test revealed that average ethanol intake was significantly decreased in the

METH-CA group during day 1 of extinction as compared to the Saline-Saline group ($p < 0.05$; Fig. 3a), and decreased ethanol intake was observed as compared to METH-Saline and Saline-Saline groups during days 2 through 7 of the extinction phase. No significant change in ethanol intake was observed between METH-Saline and Saline-Saline groups in all days of extinction.

We further investigated the effect of CA on ethanol preference. Two-way repeated measures ANOVA revealed a significant effect of time ($F(7, 175) = 2.832, p = 0.0081$), treatment ($F(2, 25) = 10.64, p = 0.0005$), and a no significant interaction between time and treatment ($F(14, 175) = 1.476, p = 0.1244$). Bonferroni's multiple comparisons revealed that ethanol preference was significantly decreased in the METH-CA group as compared to the METH-Saline group during days 1 through 7 of extinction ($p < 0.01–0.05$; Fig. 3b). Statistical analysis revealed that ethanol preference was significantly decreased in the METH-CA group as compared to the Saline-Saline group during days 1, 2, and 5 of extinction ($p < 0.05$; Fig. 3b). No significant change in ethanol preference was observed

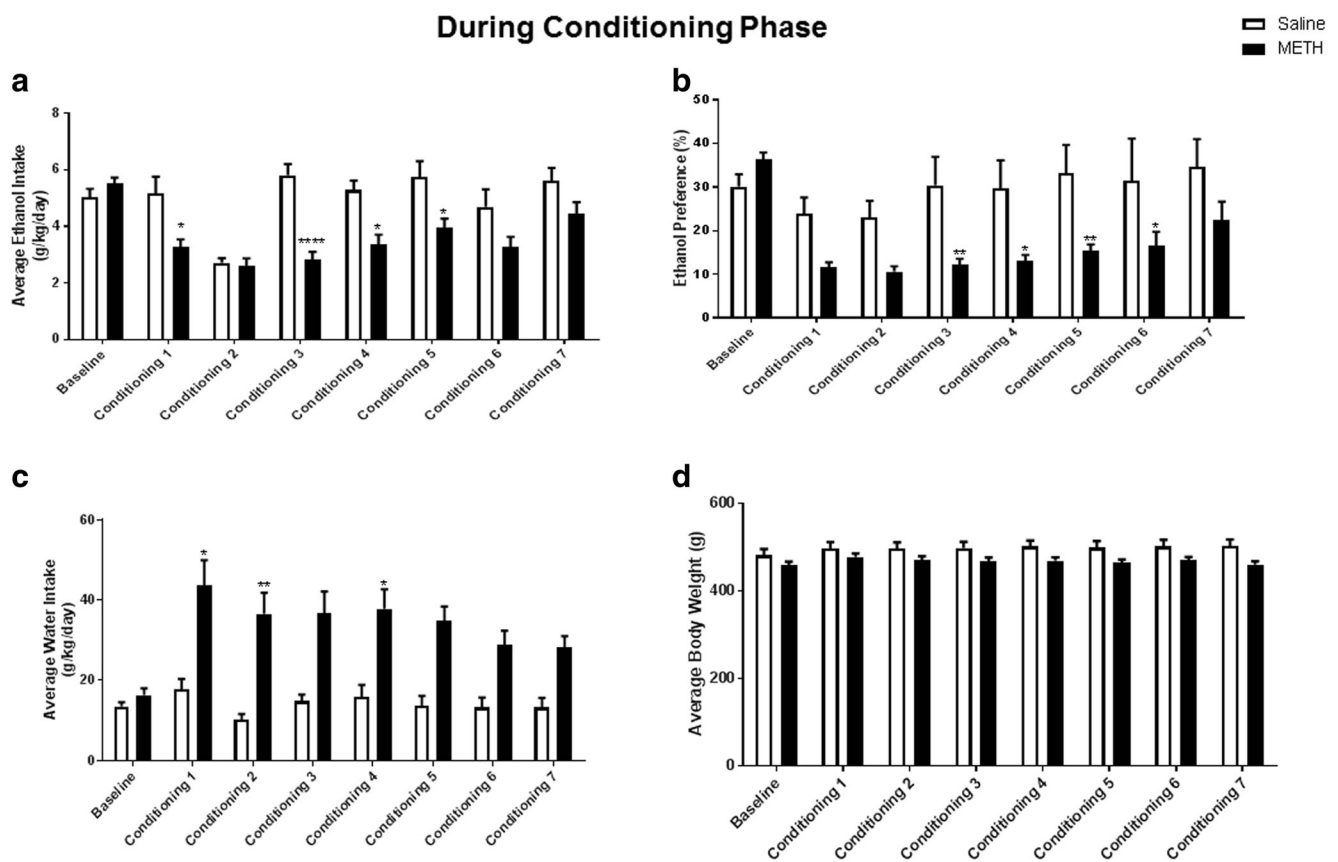


Fig. 2 Effect of METH on ethanol intake and preference during the conditioning phase. Rats had free access to three bottles containing water, 15% ethanol, and 30% ethanol for 5 weeks. Ethanol intake and preference were measured during the last 2 weeks and served as a baseline. Rats were then administered METH (2.5 mg/kg/day) or saline for 7 days during the conditioning phase, and ethanol intake (a), preference (b), water intake (c), and body weight (d) were assessed

daily. METH significantly decreased ethanol intake as compared to the saline group during days 1, 3–5 of conditioning. Ethanol preference was decreased following METH in days 3–6 of conditioning. Water intake was significantly increased in METH-treated rats as compared to saline-treated rats during days 1, 2, and 4 of conditioning. Body weight was not significantly changed following METH during the conditioning phase (* $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$ ($n = 7–21$))

between the METH-Saline and Saline-Saline groups in all days of extinction. A significant effect of time ($F(7, 175) = 4.13, p = 0.0003$), no significant effect of treatment ($F(2, 25) = 1.255, p = 0.3025$), and no significant interaction between time and treatment ($F(14, 175) = 1.066, p = 0.3920$) were revealed using two-way repeated measures ANOVA conducted on water intake. Bonferroni's multiple comparisons test did not reveal any significant change in water intake among all groups during the extinction phase ($p > 0.05$; Fig. 3c). Two-way repeated measures ANOVA conducted on body weight revealed a significant effect of time ($F(7, 175) = 5.486, p < 0.0001$), no significant effect of treatment ($F(2, 25) = 1.722, p = 0.1992$), and no significant interaction between time and treatment ($F(14, 175) = 1.352, p = 0.1815$). Bonferroni's multiple comparisons did not reveal any significant change in body weight among any groups during the extinction phase ($p > 0.05$; Fig. 3d).

Conditioned Place Preference

Effect of CA on CPP

The effects of CA were tested in control animals using the CPP paradigm. Two-way repeated measures ANOVA revealed no significant effect of time ($F(1, 6) = 0.02707, p = 0.8747$), no significant effect of chamber ($F(1, 6) = 0.04274,$

$p = 0.8431$), and no significant interaction between time and chamber ($F(1, 6) = 1.397, p = 0.2820$) (Fig. 4a).

Effect of CA on METH-Induced Reinstatement Using CPP Paradigm

METH-Saline-Treated Group Two-way repeated measures ANOVA revealed no significant effect of time ($F(3, 24) = 1.005, p = 0.4077$), no significant effect of chamber ($F(1, 8) = 1.514, p = 0.2534$), and significant interaction between time and chamber ($F(3, 24) = 10.39, p = 0.0001$). Newman-Keuls, multiple comparisons test, showed a significant increase in time spent following conditioning in the METH-paired chamber as compared to preconditioning ($p < 0.05$) and saline-paired chamber in the post-conditioning test ($p < 0.01$; Fig. 4b). A significant decrease in time spent in the METH-paired chamber was revealed following extinction training as compared to post-conditioning ($p < 0.01$; Fig. 4b). METH increased time spent in the METH-paired chamber as compared to the extinction phase ($p < 0.05$). In addition, METH increased time spent in the METH-paired chamber as compared to the saline-paired chamber ($p < 0.05$) in the reinstatement phase.

METH-CA-Treated Group Two-way repeated measures ANOVA revealed a significant effect of time ($F(3, 33) =$

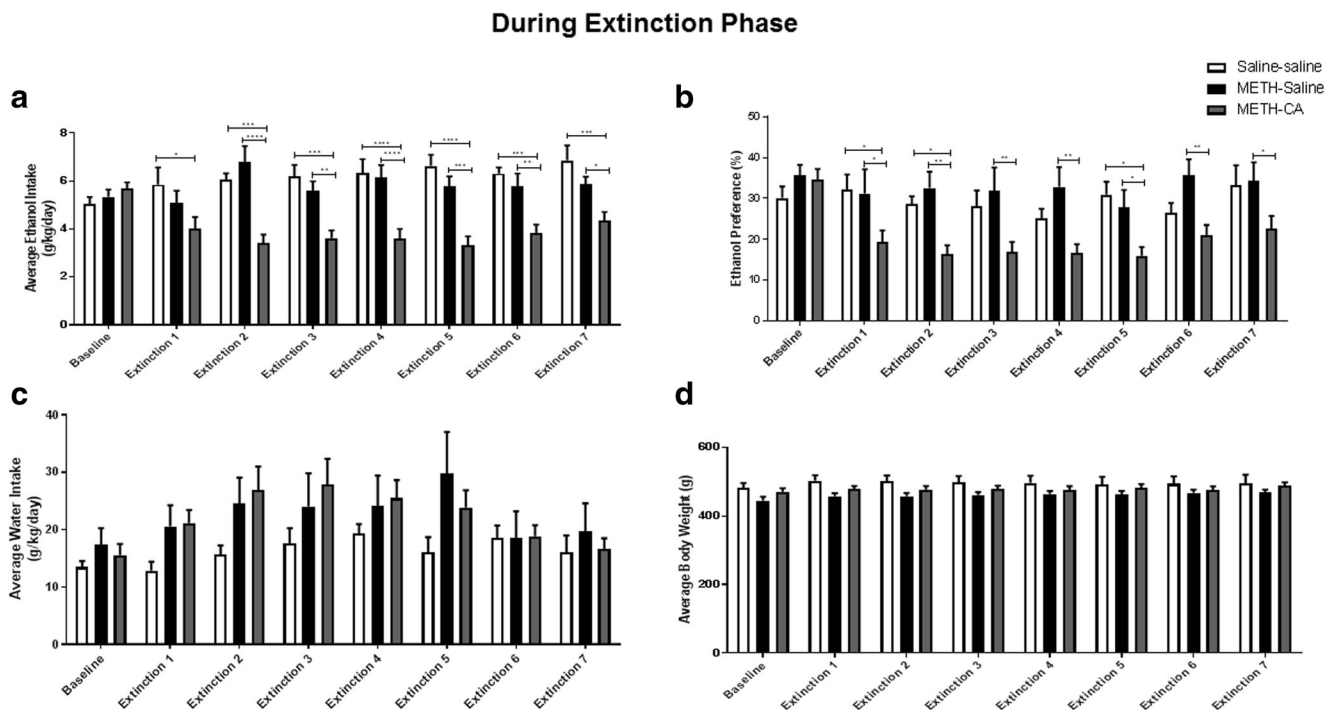


Fig. 3 Effect of CA on ethanol intake and preference during the extinction phase. Rats continued to have free access to three bottles containing water, 15% ethanol, and 30% ethanol. Rats received either CA (5 mg/kg/day) or saline for 7 days during the extinction phase. Ethanol intake (a), preference (b), water intake (c), and body weight (d)

were measured daily during the extinction phase. CA significantly decreased ethanol intake and preference during days 1–7 of the extinction phase. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. ($n = 7-12$)

2.066, $p = 0.1236$), no significant effect of chamber ($F(1, 11) = 2.401$, $p = 0.1495$), and significant interaction between time and chamber ($F(3, 33) = 10.56$, $p < 0.0001$). Newman-Keuls multiple comparisons showed a significant increase in time spent following conditioning training in the METH-paired chamber as compared to preconditioning ($p < 0.05$) and saline-paired chamber in post-conditioning ($p < 0.001$; Fig. 4c). A significant decrease in time spent in the METH-paired chamber was revealed following extinction training and reinstatement as compared to post-conditioning ($p < 0.001$ and $p < 0.01$, respectively; Fig. 4c). Time spent in the METH-paired chamber was not significantly changed following reinstatement as compared to the extinction or saline-paired chamber ($p > 0.05$).

Locomotor Activity

Two-way repeated measures ANOVA revealed no significant effect of time ($F(1, 32) = 1.223$, $p = 0.2771$), significant effect of treatment ($F(2, 32) = 15.73$, $p < 0.0001$), and significant interaction between time and treatment ($F(2, 32) = 8.657$, $p = 0.0010$). A significant decrease in locomotor activity was

revealed at baseline in Ethanol-Saline and Ethanol-METH groups as compared to the control group ($p < 0.0001$; Fig. 4d). Newman-Keuls multiple comparisons showed a significant increase in locomotor activity following 7 days conditioning treatment with METH as compared to saline treatment in ethanol-exposed rats ($p < 0.001$; Fig. 4d). A significant decrease in locomotor activity was revealed between the Ethanol-Saline group as compared to the Water control group ($p < 0.0001$; Fig. 4d). No significant change in locomotor activity was revealed between the METH-Saline group and control group following conditioning. Importantly, CA did not change locomotor activity following 7 days treatment with this drug, mean distance traveled \pm SEM in post-conditioning = 56.16 ± 4.57 m as compared to baseline = 50.46 ± 5.39 m (paired t -test, $t(6) = 1.662$, $p = 0.1477$).

Determination of the Expression of GLT-1, xCT, GLAST, and mGluR2/3 in the NAC Shell and Core As Well as the dmPFC

GLT-1 Expression We investigated the effect of ethanol, METH, and CA on GLT-1 expression in the dmPFC and

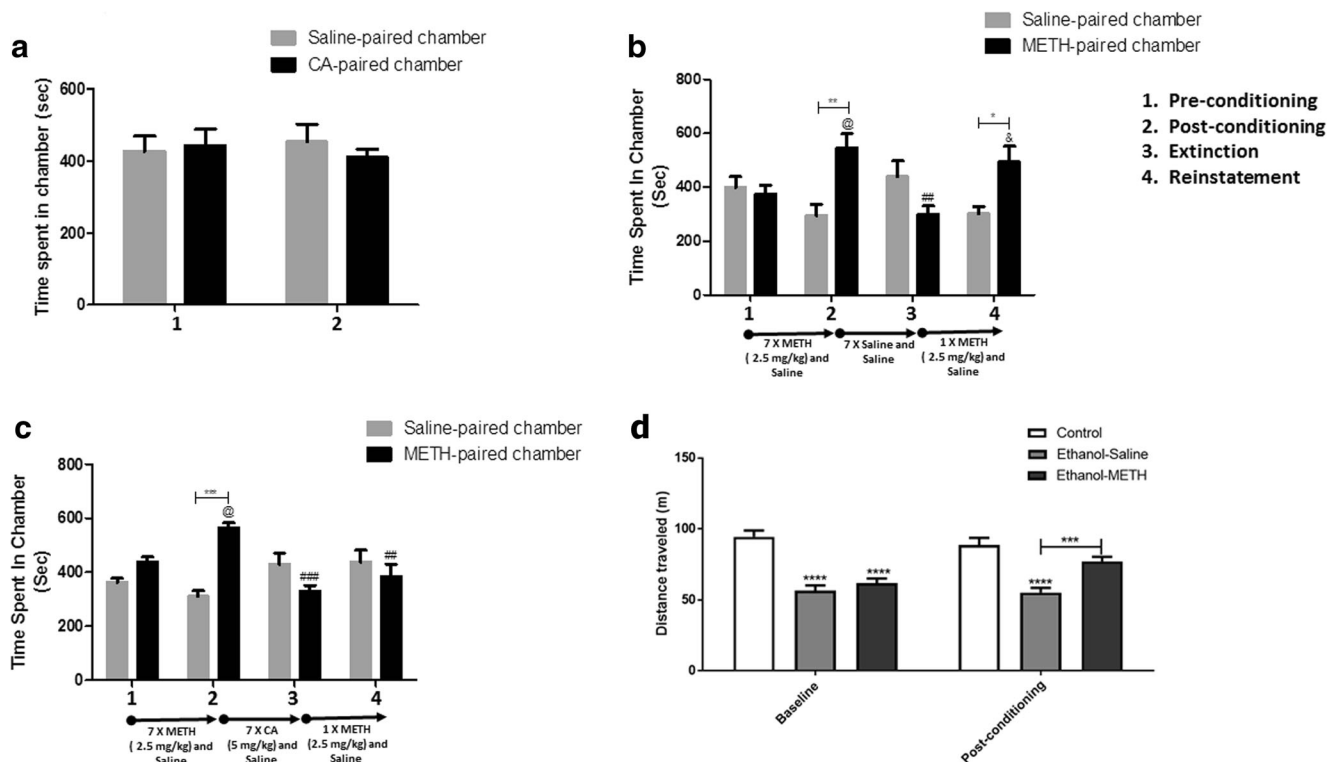


Fig. 4 Time spent and locomotor activity. Time spent in each conditioning chamber during different CPP phases in CA administered alone (a), METH-Saline group (b), and METH-CA group (c). Time spent in the CA-paired chamber was not significantly changed following 7 days treatment with CA (a). Time spent in the METH-paired chamber was significantly increased in reinstatement as compared to extinction training in METH-Saline-treated rats (b). CA blocked the reinstatement effect of METH (c). Distance traveled for rats during the baseline and after the conditioning with METH (d). Locomotor activity was lower in

ethanol groups during the baseline as compared to the control group. Following the conditioning with METH, the locomotor activity was increased as compared to the saline group. No significant difference in distance traveled between the control group and Ethanol-METH group following conditioning ($n = 9-12$ for each group). Values shown as means \pm S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ (@ $p < 0.05$) as compared to preconditioning, (# $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$) as compared to post-conditioning, while (& $p < 0.05$) as compared to the extinction phase)

NAc shell and core following the reinstatement test. One-way ANOVA revealed a significant main effect of treatment in the NAc shell ($F(3, 24) = 7.866, p = 0.0008$; Fig. 5a), but not in the core ($F(3, 24) = 0.5286, p < 0.6669$; Fig. 6a) or dmPFC ($F(3, 24) = 0.8643, p = 0.4731$; Fig. 7a). Newman-Keuls multiple comparisons showed a significant increase in the expression of GLT-1 in METH-CA-treated rats compared to METH-Saline-treated rats in the NAc shell ($p < 0.05$; Fig. 5a). However, statistical analyses showed a significant downregulation in GLT-1 expression in the Saline-Saline and METH-Saline groups compared to the control group in the NAc shell ($p < 0.05$ and $p < 0.001$, respectively; Fig. 5a). No significant difference in GLT-1 expression was revealed between the METH-CA group and the control group.

xCT Expression We investigated xCT expression in the NAc shell and core, as well as dmPFC, following the reinstatement test. One-way ANOVA revealed a significant main effect in the NAc shell ($F(3, 20) = 4.831, p = 0.0109$, Fig. 5b), but not

in the NAc core ($F(3, 24) = 0.2322, p < 0.8730$; Fig. 6b) or dmPFC ($F(3, 24) = 1.15, p = 0.3491$; Fig. 7b). Newman-Keuls multiple comparisons showed a significant increase in the expression of xCT in METH-CA-treated rats compared to the Saline-Saline-treated rats in the NAc shell ($p < 0.05$; Fig. 5b). However, statistical analyses showed significant downregulation in xCT expression in the Saline-Saline group compared to the control group in the NAc shell ($p < 0.05$; Fig. 5b). No significant difference in xCT expression was revealed between the METH-CA and METH-Saline groups as compared to the control group.

GLAST Expression We further tested the effect of ethanol, METH, and CA on GLAST expression in the NAc shell and core, and dmPFC following the reinstatement test. One-way ANOVA revealed a non-significant main effect of treatment in the NAc shell ($F(3, 24) = 0.7276, p = 0.5456$; Fig. 5c), NAc core ($F(3, 20) = 1.096, p = 0.3740$; Fig. 6c), and dmPFC ($F(3, 20) = 0.2637, p = 0.8507$; Fig. 7c).

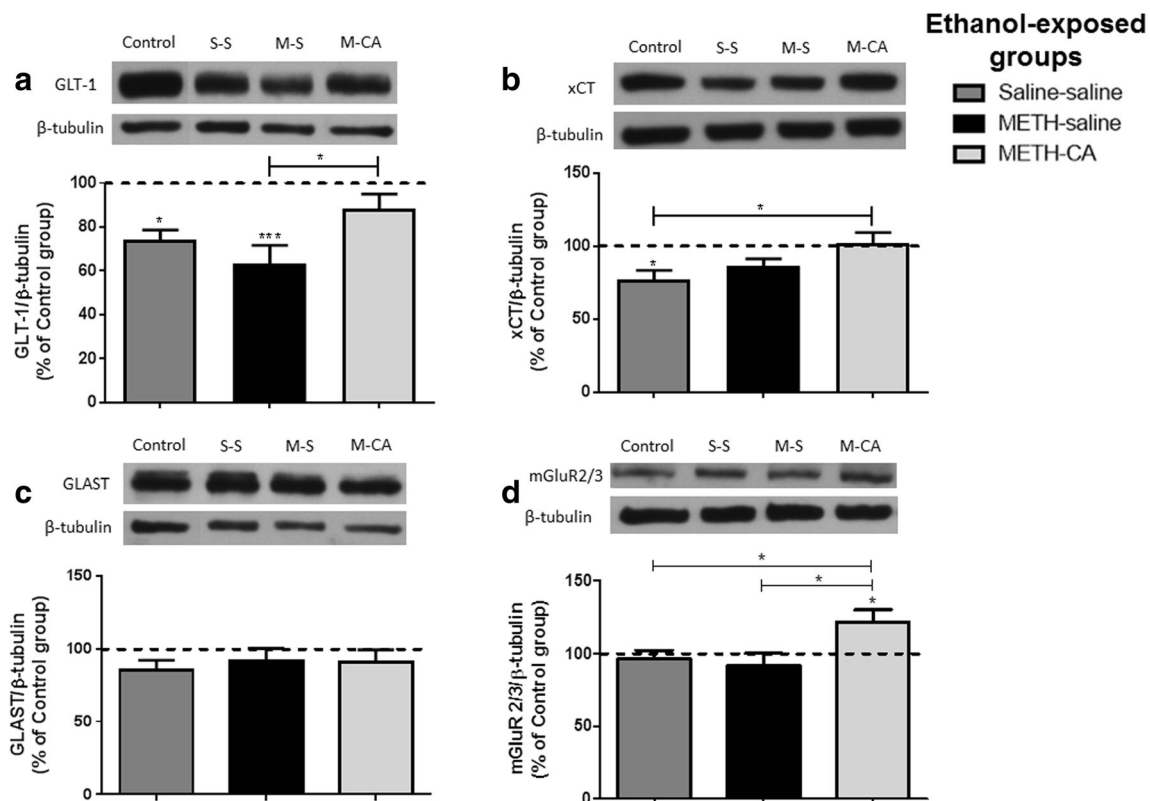


Fig. 5 Effects of ethanol, METH (2.5 mg/kg), and CA (5 mg/kg) on the expression of GLT-1 (a), xCT (b), GLAST (c), and mGluR2/3 (d) in the NAc shell. Quantitative analysis revealed a significant increase in the ratio of GLT-1/ β -tubulin in METH-CA-treated rats compared to the METH-Saline-treated rats. A significant downregulation of GLT-1 expression was revealed in the METH-Saline and Saline-Saline (exposed to ethanol without METH) groups compared to the control water group. A significant increase in the ratio of xCT/ β -tubulin was

revealed in the METH-CA group compared to the Saline-Saline (exposed to ethanol without METH) group. Significant downregulation of xCT expression was revealed in Saline-Saline-treated group compared to the control water group. A significant increase in the ratio of mGluR2/3/ β -tubulin was revealed in the METH-CA group compared to METH-Saline, Saline-Saline, and control groups. No significant difference in GLAST expression was revealed among all tested groups in the NAc shell. * $p < 0.05$. Values are shown as means \pm S.E.M. $n = 6-7$ for each group

mGluR2/3 Expression We investigated the effect of ethanol, METH, and CA on mGluR2/3 expression in the dmPFC, and NAc shell and core following reinstatement test. One-way ANOVA revealed a significant main effect in the NAc shell ($F(3, 24) = 3.996, p = 0.0193$; Fig. 5d), dmPFC ($F(3, 20) = 3.116, p = 0.0492$; Fig. 7d), but not in the NAc core ($F(3, 24) = 0.294, p = 0.8293$; Fig. 6d). Newman-Keuls multiple comparisons showed a significant increase in the expression of mGluR2/3 in the METH-CA-treated rats compared to the control group in the NAc shell ($p < 0.05$; Fig. 6d) and dmPFC ($p < 0.05$; Fig. 7d). Moreover, statistical analyses showed a significant increase in mGluR2/3 expression in the METH-CA group compared to Saline-Saline and METH-Saline groups in the NAc shell ($p < 0.05$; Fig. 5d), but not in dmPFC ($p > 0.05$; Fig. 7d).

Discussion

In this study, we showed that chronic ethanol exposure caused significant downregulation in GLT-1 and xCT expression in the NAc shell. Of note, GLT-1 and xCT are responsible for clearing and maintaining extracellular glutamate to be at physiological levels in several brain regions implicated in drug dependence (Baker et al. 2002; Danbolt 2001; Rothstein et al. 1995). In line with previous reports, GLT-1 and xCT

were downregulated in the NAc following exposure to various drugs of abuse, including ethanol and METH (Alhaddad et al. 2014; Althobaiti et al. 2016; Das et al. 2015; Fischer et al. 2013; Goodwani et al. 2015; He et al. 2014; Knackstedt et al. 2009; Sari and Sreemantula 2012; Sari et al. 2013). Importantly, NAc is a well-known brain region involved in the rewarding and reinforcing effects of drugs of abuse (Bardo 1998; Koob and Bloom 1988; Wise and Rompré 1989). Deficits in glutamate clearance in the NAc have been found to be associated with chronic drug use and drug-seeking behavior (Das et al. 2015; Fujio et al. 2005; Knackstedt et al. 2010; Melendez et al. 2005). Importantly, studies showed that chronic ethanol drinking decreased the expression of GLT-1 and xCT in the NAc and other brain regions, and restoring the expression of these proteins was associated with reduction in ethanol drinking (Aal-Aaboda et al. 2015; Alhaddad et al. 2014; Das et al. 2015; Sari and Sreemantula 2012). In accordance with the present study, CA treatment restored GLT-1 and xCT expression levels in the NAc shell and decreased ethanol intake. This is also in accordance with our previous reports that demonstrated decrease in ethanol intake following CEF treatment, which was associated with upregulation of GLT-1 in the NAc (Alhaddad et al. 2014; Sari et al. 2011, 2013). Importantly, CA has some advantages over CEF such as lack of antibiotic activity, oral availability, relative safety, and the ability to cross the blood-brain barrier (Münch et al.

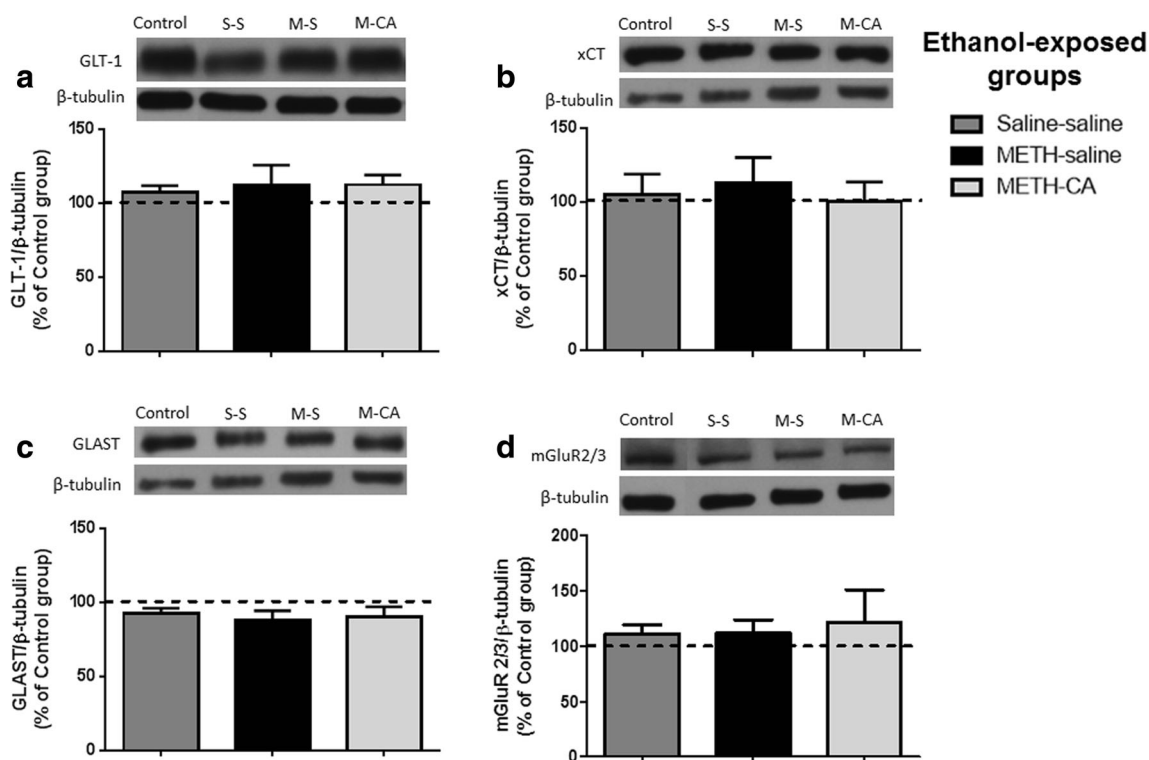


Fig. 6 Effects of METH (2.5 mg/kg) and CA (5 mg/kg) on the expression of GLT-1 (a), xCT (b), GLAST (c), and mGluR2/3 (d) in the NAc core. The quantitative analysis did not reveal any significant change in the

expression of GLT-1, xCT, GLAST, and mGluR2/3 among all tested groups in the NAc core. Values are shown as means \pm S.E.M. $n = 6-7$ for each group

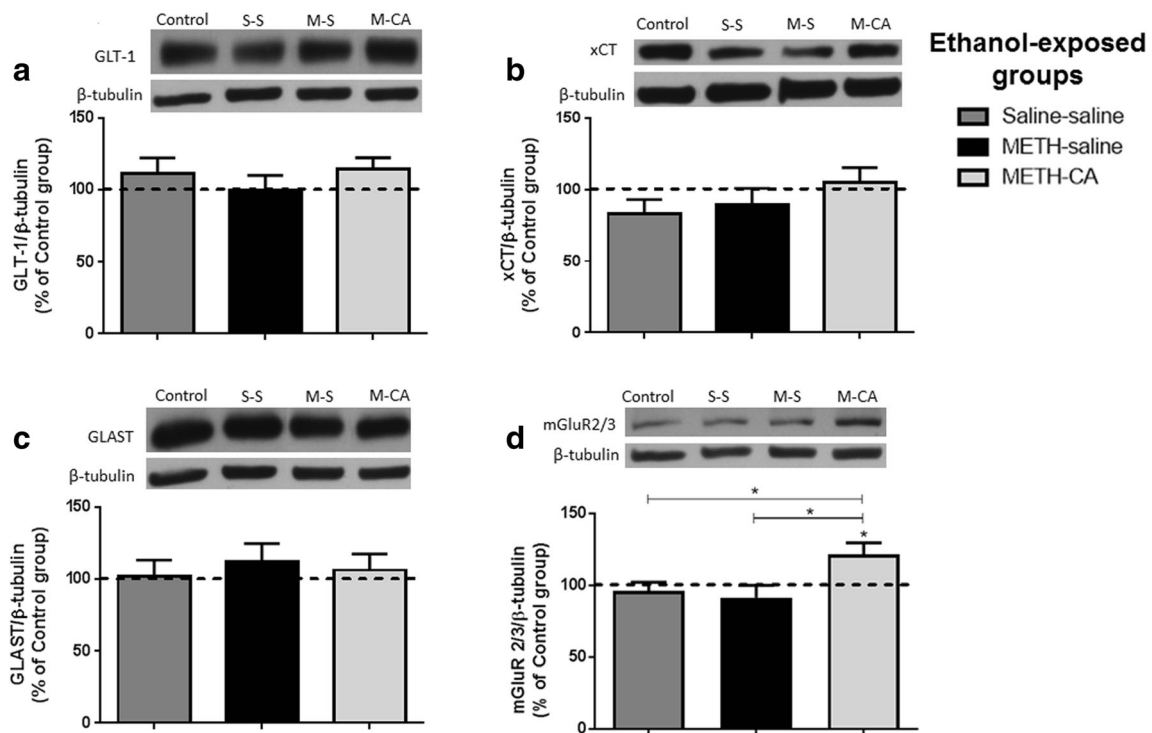


Fig. 7 Effects of METH (2.5 mg/kg) and CA (5 mg/kg) on the expression of GLT-1 (a), xCT (b), GLAST (c), and mGluR2/3 (d) in dmPFC. Quantitative analysis revealed a significant increase in the ratio of mGluR2/3/β-tubulin in the METH-CA group compared to the control

group. No significant difference in GLT-1, xCT, or GLAST expression was revealed among all tested groups in dmPFC. * $p < 0.05$. Values are shown as means \pm S.E.M. $n = 6-7$ for each group

1981; Nakagawa et al. 1994). These advantages suggest that CA might be a potential compound for the management of drug dependence.

In this study, we revealed that the reduction in the expression of GLT-1 and xCT following chronic ethanol exposure is specific to the shell rather than the core subregion of the NAc. This suggests the important role of the NAc shell in mediating the rewarding and reinforcing effects of ethanol. Indeed, ethanol exposure has been shown to increase the release of dopamine in the NAc shell of rats selectively bred to prefer ethanol more than rats bred to avoid ethanol (Bustamante et al. 2008). Moreover, a recent report showed that blocking dopamine D1 receptor in the NAc shell but not in the core decreased ethanol-seeking behavior in P rats (Hauser et al. 2015). Moreover, the reinforcing effect of ethanol has been shown to be mediated through the activation of dopaminergic receptors in the NAc shell, but not the NAc core (Ding et al. 2015). However, we have previously shown that ethanol drinking for 6 weeks caused downregulation of GLT-1 expression in both the NAc shell and core (Sari et al. 2013). In this study, rats were exposed to chronic ethanol drinking for a more extended period (8 weeks). This might indicate possible neuroadaptation that might have occurred in the core, but not the NAc shell, to the effect of ethanol on GLT-1 expression. We suggest that GLT-1 is more sensitive to the effect of ethanol in the shell subregion of the NAc than the core.

However, further studies are still needed to examine the subregional differences of the NAc as well as other brain regions. On the other hand, the expression of GLAST was not altered in any brain region tested, which is in line with previous reports (Alhaddad et al. 2014; Hakami et al. 2016). Studies suggested that GLAST is highly expressed in the cerebellum and retina, while GLT-1 and xCT predominantly regulate glutamate homeostasis as compared to GLAST in forebrain regions, including the NAc (Baker et al. 2002; Danbolt 2001; Lehre et al. 1997; Rothstein et al. 1994, 1995). This might be a key factor in mediating the effects of ethanol on the expression of these transporters.

Moreover, chronic ethanol exposure caused reduction in the locomotor activity of rats as compared to the control group, which is consistent with the sedative effect of ethanol. Interestingly, METH reversed the negative effect of ethanol drinking on locomotor activity, which corroborates with a recent clinical study (Kirkpatrick et al. 2012). METH stimulant effect resulted in an improvement of the detrimental effect of ethanol on locomotor activity. This might indicate that the high prevalence of METH and ethanol co-abuse found in clinical studies may involve some degree of compensation or self-medication (for a review, see ref. (Althobaiti and Sari 2016)).

On the other hand, we found that METH initially caused reduction followed by gradual restoration of ethanol intake.

The cause of this effect is unclear. However, it might be due to possible interactions between METH and ethanol on dopaminergic pathways in the NAc. Indeed, *in vivo* microdialysis studies have reported an increase in dopamine release in the NAc following oral ethanol as well as METH self-administration (Jang et al. 2017; Lominac et al. 2012; Weiss et al. 1993). Dopamine in the NAc is known to regulate motivated behavior and reward to drugs of abuse (for a review, see ref. (Ikemoto and Panksepp 1999)). It is possible that METH initially increases dopamine release in the NAc with the associated motivational state, which in turn may decrease ethanol-seeking behavior. This initial effect might be followed by gradual decreases in dopamine release following METH exposure, which consequently may lead to an increase in ethanol drinking to reach the hedonic effects. Further studies are needed to investigate the neurochemical basis of this effect using the no-net flex microdialysis technique.

Importantly, we showed for the first time that CA blocked the reinstatement of METH. The attenuating effect of CA on METH-induced reinstatement is unlikely caused by CA-induced changes in CPP preference or locomotor activity. Indeed, CA did not affect the CPP and locomotor activity when the drug is administered alone. Expression of GLT-1 and xCT was restored following CA treatment in the NAc shell which might be, in part, the underlying mechanism in blocking the reinstatement of METH-seeking and reducing ethanol drinking. It has been reported that CA is capable of increasing the expression of GLT-1 in the NAc (Kim et al. 2016). In this study, we showed that CA increased the expression of xCT and restored the GLT-1 expression in the shell subregion of the NAc but not in the core. Moreover, CA treatment increased the expression of mGluR2/3 in both the NAc shell and dmPFC. It is important to note that the elevation of mGluR2/3 expression in the PFC was associated with reduction in ethanol seeking (Meinhardt et al. 2013). Activation of mGluR2/3 in the NAc shell but not the NAc core blocked context-induced reinstatement of heroin seeking (Bossert et al. 2006). Interestingly, activation of mGluR2/3 in the NAc was shown to block amphetamine-induced release of dopamine (Pehrson and Moghaddam 2010). Together, restoring the expression of GLT-1 and xCT in the NAc shell as well as increasing the expression of mGluR2/3 in the NAc shell and dmPFC can improve the glutamatergic homeostasis, which in part attenuate ethanol drinking as well as METH reinstatement. It is important to note that the effects of METH alone on these glutamate transporters and receptors were not examined in this study. Further studies are needed to examine the neurochemical changes related to METH exposure alone and any neuroprotective effects of CA treatment.

In conclusion, our findings showed for the first time that CA attenuated reinstatement to METH-seeking and decreased ethanol drinking behavior. These behavioral effects of CA are most likely due to its effect on GLT-1, xCT, and mGluR2/3

expressions in the NAc shell and dmPFC. These findings suggest that CA could be a potential safe therapeutic agent for management of drug dependence and relapse.

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Author Contributions YSA participated in study design and conceptualization, drafted and revised the manuscript, and collected and analyzed the data. FSA participated in study design, collected the data, and helped with the editing of the manuscript. AYH collected the data and helped with the editing of the manuscript. AH performed the locomotor activity experiments and helped with editing the manuscript. YS conceptualized and designed the study, critically revised the manuscript for intellectual content, and approved the final version of the manuscript.

Compliance with Ethical Standards

Animal experimental procedures were approved by the Institutional Animal Care and Use Committee of The University of Toledo in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institutes of Health and the Guide for the Care and Use of Laboratory Animals.

Conflict of Interest The authors declare that they have no conflict of interest.

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